EX. 4

Detection of inhibitors in milk by microbial tests A review

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Summary

The demands concerning microbial inhibitor tests were subjected to marked changes during the last decades: It started with the claim of being able to detect contaminated milk which might cause problems during fermentation processes ('technological safety'). Due to the present day attention drawn to toxic and allergic hazards for numerous antimicrobials Maximum Residue Limits (MRLs) or safe/tolerance levels are fixed ('toxicological safety'). This means a variety of demands underlying permanent changes with respect to the 'detection pattern' which cannot be fulfilled by a single test. Within an Integrated Detection System microbial inhibitor tests play an important role as screening methods for those antimicrobials which can be detected with satisfactory sensitivities. This paper deals with some features of microbial inhibitor tests such as detection limits, performance, susceptibility for interference factors, qualitative determination and standardization.

Introduction

Antimicrobials were applied in the treatment of dairy cows almost as soon as they had been developed [1–3] and soon after their introduction the adverse effect of using milk from antibiotic-treated cows for the production of fermented milk products became evident [4–9]. The economic losses due to fermentation failures and the influence of antibiotics on the results of reduction tests [10] were the reasons for developing screening methods for the detection of contaminated milk. The methods for the detection of antibiotic residues in milk were based mainly either on microbial agar diffusion tests developed for the assay of antibiotics used in clinical medicine or on inhibition of acid production/coagulation by starter organisms [11–14]. Table 1 summarizes test principles and test microorganisms applied [13, 15–29] and it becomes evident, that the main basic research in this area was already done during the period 1947–1961. Advances since that time had the main aim to improve sensitivities for an increasing number of antimicrobials, rapidity, ease of performance, standardization and accuracy.

According to the legislation of one state in the north of Germany (Schleswig-Holstein) starting in 1964 farm milk was regularly tested and paid according to its ability to produce acid and since 1972 the Brilliantblackreduction test (BRT) with *Bacillus stearothermophilus* as test microorganism has been applied [30].

Descriptions of inhibitor tests applied and/or discussed today are collected by Group E 503 (former Group E 47) of the International Dairy Federation (IDF) and are published in IDF-Bulletin 258 [31]. Intercomparison studies within this group of experts have revealed that in most countries agar diffusion tests with *B. stearothermophilus* in various forms – disc assay, Delvo-test, BRT- and acidification tests with *Streptococcus thermophilus* are most frequently used today [32].

Table 1. Test principles and test microorganisms of microbial inhibitor tests for milk

Principle	Test microorganism	References
Morphological changes	*S. cremoris* *S. thermophilus*	Whitehead and Cox, 1956 [15] Liska, 1960 [16]
Agar diffusion tests		
Cylinder Plate Well assay Disc assay	*Staph. aureus* *Sarcina lutea* *B. subtilis* *Mc. pyogenes* *B. cereus* *B. mycoides*	Juncher et al., 1950 [17] Welsh et al., 1948 [18] Jacque and Steeg, 1953, [20]
Agar diffusion tests with indicator (triphenyltetrazoliumchloride (TTC), brillantblack, bromcresolpurple)	*B. stearothermophilus*	Igarashi et al., 1961 [21] Kraack and Tolle, 1967 [22] Van Os et al., 1975 [23]
Agar diffusion test with trimethoprim	*B. stearothermophilus* *Mc. luteus* *B. megaterium*	Gudding, 1976 [24]
Inhibition of acid fermentation/coagulation with indicator (methylene blue, resazurin, TTC, bromcresolpurple, litmus)	*S. cremoris* Starter culture *L. bulgaricus* *S. lactis* *S. thermophilus* *B. cereus var. mycoides* *B. mesentericus*	Friedman and Epstein, 1951 [25] Ruehe, 1950 [14] Sanchez and Lamensans, 1947 Berridge, 1953 [26] Schipper and Peterson, 1955 [13] Neal and Calbert, 1955 [28] Ullberg, 1952 [29]

Changing demands towards microbial inhibitor tests

The demands concerning microbial inhibitor tests have been subjected to marked changes during the last decades: It started with the claim that by application of the inhibitor test milk contaminated with antimicrobial residues – in the beginning only penicillin – which might give rise to problems during fermentation processes can be excluded. At-

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Table 2. Maximum Residue Limits (Codex, EU) and safe/tolerance levels (FDA) of antimicrobial residues in milk [µg/kg]

Substance (-group)	MRL Codex	MRL EU (1)	Safe/tolerance (2)	Substance (-group)	MRL Codex	MRL EU (1)	Safe/tolerance (2)
β-Lactams				*Macrolides*			
Penicillin	4	4(5)	5/0	Erythromycin		40(6)	50/0
Ampicillin		4(5)	10/10	Spiramycin	100	200(5)	
Amoxicillin		4(5)	10/10	Tylosin		50(6)	50/50
Cloxacillin		30(5)	10/10				
Dicloxacillin		30(5)		*Aminoglycosides*			
Oxacillin		30(5)		Gentamicin	100(9)		30/0
Ceftiofur	100	100(6)	50(7)/1000(8)	Neomycin	500(9)		150/150
Cephapirin			20/20	DH/Streptomycin	200(9)		125/0
Tetracyclines (3)		100(6)		*Others*			
Chlortetracycline			30/0	Dapsone		0(5)	
Oxytetracycline	100		30/0	Chloramphenicol		0(5)	0/0
Tetracycline			80/0	Novobiocin			100/100
				Spectinomycin	200(9)	200(6)	30/0
				Trimethoprim		50(6)	
Sulfonamides (3)		100(6)					
Sulfadimidine (4)	25		10/0				
Sulfadimethoxine			10/10				
Sulfamerazine			10/0				
Sulfathiazole			10/0				
Sulfadiazine			10/0				

(1) EU-Regulations 675/92; 3093/92; 2901/93, 3436/93; 1430/94; (2) CFR 21 and CVM correspondence; (3) All substances belonging to the group; (4) = Sulfamethazine; (5) Final; (6) Preliminary; (7) Parent drug; (8) Total parent and metabolite; (9) Compounds on agenda

tention drawn to toxic and allergic hazards caused by antimicrobial residues started at the beginning of the 60s and led to a first fixed determination level of one substance: According to EEC-Directive 85/397 [33] only milk with less than 0.004 µg penicillin/ml (= 4 µg/kg) and other residues not detectable was allowed to be used as milk for consumption, which meant in practice that the inhibitor tests applied were adjusted to a detection limit of 4 µg penicillin/kg without detailed considerations of the detection of other antimicrobials. Today for numerous antimicrobials Maximum Residues Limits (MRLs) (Codex, EU) or FDA-safe/tolerance levels are fixed [34]. The status of the MRLs is different – some are final, others are preliminary –, the number of substances will increase in the future, and in some cases the MRLs and safe/tolerance levels of the same substance differ, as summarized in Table 2.

Position of microbial inhibitor tests within an integrated detection system

From Table 2 – which cannot be regarded as complete as in different countries, for example Canada, partly different substance/concentration combinations are valid [35] – it becomes evident that there will be no single test which can fulfil this variety of demands underlying permanent changes. As the incidence of residues of antimicrobials in milk in most areas is less than 1% there exists the need for an easy and cheap-to-perform test which detects a broad spectrum of antimicrobials for screening purposes. As demonstrated schematically in Fig. 1 microbial tests play an important role in such screening methods. The interpretation of this graph is that milk samples are screened by microbial inhibitor tests and, depending on further requirements – quality payment, self control in the dairy, food inspection – positive samples are further analysed by more

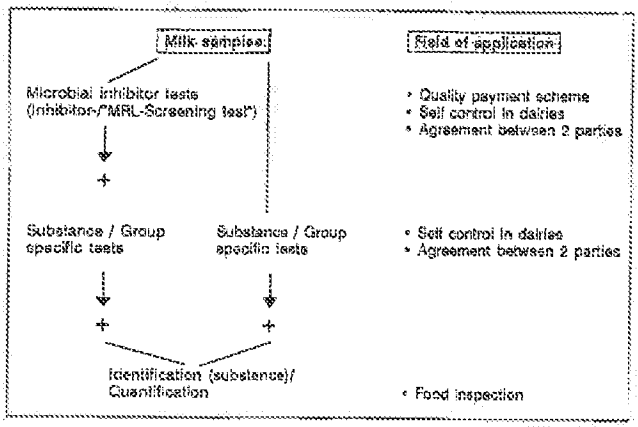

Figure 1. Position of microbial inhibitor tests within an integrated detection system

sophisticated methods which allow identification and quantification. As there are no available microbial inhibitor tests with satisfactory detection limits for all antimicrobials in question, for example chloramphenicol, it is necessary to start examinations with specific and sensitive tests to detect those residues.

Features of microbial inhibitor tests

The ideal screening test yields no false negatives at the level of concern and a positive result indicates that follow-up action is required. In Table 3 features of microbial inhibitor tests are summarized which characterize them as 'ideal' as screening methods within an integrated detection system.

Table 3. Features of 'ideal' microbial inhibitor tests

Dectection	— broad variety of antimicrobials
	— limits according to requirements for example MRLs
Performance	— easy, for example no educated persons, no sophisticated equipment
	— cheap
	— suitable for mechanized mass analysis
	— fast results
Susceptibility for interference factors	
	— low due to sample composition, for example lysozym content, microflora
	— low due to test procedure
Possibilities of identification	— easy to perform, for example β-lactamase test
Standardization	— low variability within and between batches
	— low variability within and between laboratories

The idea is to have available methods by which a broad variety of antiinfectives are detected with sensitivities which correspond exactly with the demands, as for example within the EU MRL-concept. The inevitable facts which have to be taken into account within this context are demonstrated schematically in Fig. 2. Each test is characterized by a specific 'detection pattern', with the *detection* of a limited spectrum of antiinfectives at various levels with respect to the requirements: There are assays which detect

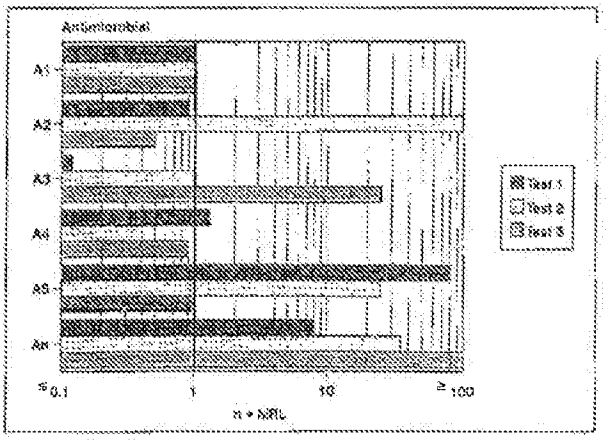

Figure 2. 'Detection pattern' of different microbial inhibitor tests for antimicrobials A1, A2 ... An. Detection limits expressed in n-fold MRL-concentration

several substances at the concentration levels required whereas other substances — even of the same group of antiinfectives such as for example β-lactam antibiotics — are detected less or more sensitive. Even tests of very similar design will differ in their 'detection patterns', which makes it inevitable that results can differ when milk samples are analysed by different methods.

The stringency of detection limits evaluated depends on the kind of antimicrobial under study due to different modes of action of the antimicrobials on the test microorganisms. Fig. 3 demonstrates exemplary dose-response curves of antimicrobials A1 and A2. For some antimicrobials (for example penicillin) there is only a narrow concentration range where test results are evaluated differently (A1) whereas other antimicrobials (for example tetracyclines) lead to ambiguous results spread over a broad range of concentrations (A2). This means that in milk samples with positive test results the probability that the concentration in the milk sample is below the detection limit is less pronounced with antimicrobial A1 than with A2.

There are numerous test kits available which allow easy *performance* of analyses without sophisticated instruments and educated staff, they are relatively cheap and in a more or less mechanized way for mass analysis such as for example BRT, Charm AIM, Delvo, Valio T101. Due to the Milk Quality Ordinance, farm milk in Germany is examined regularly by the BRT. This means 5—6 mill analyses per year. The price per sample calculated by one control laboratory is about 0.65 DM.

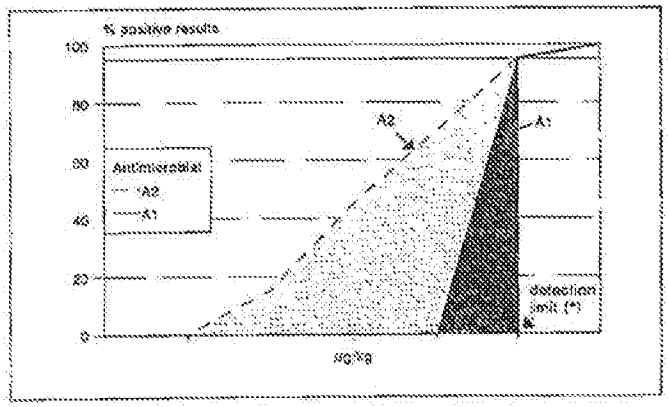

Figure 3. Dose [µg/kg]-response [% positive results]-curves of two different antimicrobials (A1, A2)
(*) Detection limit defined as concentration where 95% of test results were evaluated as positive

The time elapsing between sample analysis and the availability of the test results for the most often applied inhibitor tests varies between about 2—4 h with *B. stearothermophilus* and *S. thermophilus* tests and 16—24 h with mesophilic spore formers as test microorganisms. Due to the test principle — measurement of the inhibition of multiplication and/or metabolism of the test microorganism — the microbial inhibitor tests cannot serve as rapid tests when test results are required within a few minutes. Attempts to shorten analysis time, for example by ATP measurements or use of sensitive indicators, has led to minimum periods of 30—60 min [31].

Interference factors with influence on the test result can arise from

- sample composition, or
- susceptibility during test procedure.

In general it can be stated that

- the influence of the sample composition will decrease with increasing number of milkings included, and
- inhibitor tests with increasing sensitivities for the detection of a variety of antimicrobials may also have an increased susceptibility for interference factors.

The major non-specific antibacterial factors in milk — lactoferrin and lysozyme — the content of which is elevated in mastitis milk and colostrum, are discussed as reason(s) for 'false' positive results of microbial inhibitor tests. Experimental studies have shown, that the concentration of single components needed to achieve an inhibition was unphysiologically high, whereas the combination of both substances showed a synergistic effect and were even in physiological concentrations inhibitory. In order to induce positive results considerably higher concentrations were needed with agar containing inhibitor test systems than in liquid ones, which is probably due to an ion-binding effect of the agar [36].

The microflora of the test samples can be of importance with respect to

- influence on the test microorganisms, and
- metabolic activity in the sample.

In order to avoid the influence of the microflora of the sample on the test microorganism in most cases, where mesophilic test microorganisms are used in the test procedure, a heat pre-treatment of the samples is necessary in order to inactivate major parts of the milk flora. In the case of the thermophilic *B. stearothermophilus* as test microorganism the incubation of the test systems takes place at temperatures ≥50 °C — a temperature range where only minor parts of the milk flora can multiply and metabolize.

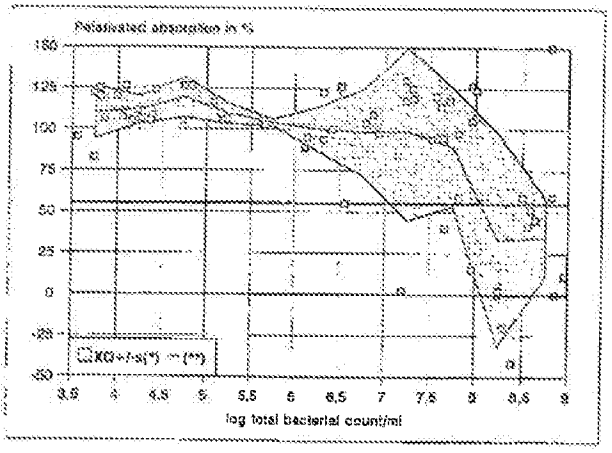

Figure 4. Influence of total bacterial count on the evaluation of milk spiked with 4 µg penicillin/kg by BRT (mod.)
(*) XQ and s within 0.5 log total bacterial count steps;
(**) ≥55% relativated absorption positive results

As demonstrated in Fig. 4, the activity of microorganisms can cause 'false' negative results in a milk sample contaminated with penicillin. The spiked milk samples were stored at 6 °C and analysed by the standard plate count method and a modified BRT with evaluation by relativated absorption in % on several subsequent days [37]. Relativated absorption values ≥55% correspond to visually evaluated positive results in the case of BRT. As can be derived from this graph, the BRT was evaluated as negative in some cases when the total bacterial count exceeded about log 6.5/ml (3 mill/ml). This is probably due to β-lactamase producing microorganisms within the flora.

From practical experience there exist scare reports that 'false' positive results were caused by mouldy feed or by nisin produced by microorganisms within the sample. In the state of its first development the BRT was relatively insensitive towards the pH-value of the sample whereas its modified form, the Blue Star test, will show questionable or positive results when the pH-value of the sample is ≤6.1. Most reports about the influence of residues of sanitizers in the sample deal with *B. stearothermophilus* tests. This test microorganism is rather insensitive towards practically applied sanitizers and is not markedly influenced by concentrations to be expected in milk samples [38].

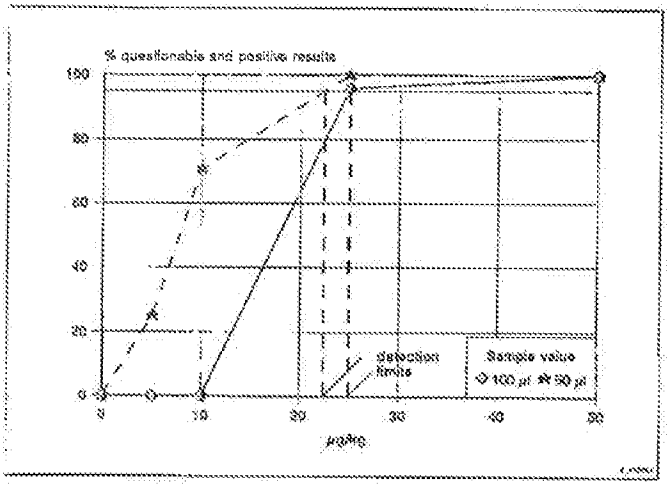

Figure 5. BR-EC: Influence of sample volume (100 and 50 µl resp.) on the detection of tylosin-visual evaluation; n = 24 per substance/concentration combination; detection limit defined as concentration where 95% of test results were evaluated as positive

Factors interfering during the test procedure may be the reagents used, incubation conditions and sample volume. Due to experience gained during a validation study of one *B. stearothermophilus* test it became evident that these factors can be of different influence in dependency on the antimicrobial under study. The influence of the sample volume on the detection limit of tylosin by the BR-EC test is demonstrated as an example in Fig. 5. It becomes evident that the dose-response-curve is markedly influenced by the sample volume, and the defined detection limits differ only slightly. Whereas the detection limit of oxacillin was not markedly influenced whether the sample volume was 100 µl or 50 µl the detection was more sensitive in the case of spiramycin and oxytetracycline, and less sensitive in the case of penicillin and dicloxacillin when 50 µl sample is used instead of 100 µl.

The conception of an 'ideal' microbial inhibitor test includes the possibility of *identification and quantification* of the inhibitory substance(s) in the sample by the simple to perform microbial inhibitor tests. In principle addition of certain substances to the inhibitor positive reacting sample and re-analysis allows the following identification [39]:

β-lactamase → β-lactam antibiotics
PABA → sulfonamides/dapsone
Cysein → streptomycin
Hydroxylamine → DH/streptomycin
$NaOCl/Na_2S_2O_3$ → tetracyclines

The latter 3 treatments were used mainly in electrophoretic methods for identification of residues followed by bioautography [31, 39]. This electrophoretic method requires much more effort compared to microbial inhibitor tests with respect to equipment, sample extraction, analysis and evaluation; it needs to be re-evaluated in relation to sensitivities required according to the MRL-concept for example. In routine testing the identification of β-lactam antibiotics and sulfonamides is applied rather often [31, 40]. In both cases a group of antimicrobials and not an individual substance can be identified, and therefore quantitative determination of a substance is excluded in the case of examining unknown samples. In order to interpret identification results it is necessary to know what amount of which substance can be detected by the procedure applied. In the case of β-lactam antibiotics, type and activity of β-lactamase used and the exposure conditions in the milk samples are of importance in the inactivation of the individual β-lactam antibiotics [39, 41]. Fig. 6 shows the inactivation of dicloxacillin by various β-lactamase preparations and activities.

For the use of microbial inhibitor tests within a testing system the *standardization* of test kit manufacture, performance and interpretation are vital prerequisites in order to obtain reliable, comparable results. The interpretation of standardization figures – which for most tests have not been examined in detail until now – have to be regarded with respect to the fact that this kind of test is based on measurements of living cells and that usually the results are obtained by subjective visual reading within 2–3 steps (negative, questionable, positive). Results of collaborative studies make it very obvious, that a lot of further effort is required with regard to standardization.

Fig. 7 shows the results for oxytetracycline (OTC) of the participating laboratories on an IDF-intercomparison study [32]. Test samples were prepared and shipped from a central place to laboratories in 15 different countries. The detection limits of various microbial inhibitor tests in relation to the claimed detection limits were investigated. It becomes evident that the claimed detection limits of OTC are in the range which were evaluated in the participating labs in the case of disc assay, Delvo-, acidification- and Valio T101-test, whereas in the case of the BRT-modifications and the Charm Farm test the claims could not be confirmed. With the exception of BRT-6 and Valio T101 the concentration range of OTC with 50%–100% positive results is rather broad, for example in the case of the Charm Farm test 50% positive results were indicated at the level of 100 µg OTC/kg whereas 100% positive results were indicated on the level of 750 µg OTC/kg.

One result of another proficiency study [42] in which split test samples were analysed with 7 different microbial inhibitor tests which were prepared at one place and shipped to the 9 participating, experienced labs was that, including all tests and samples minimum and maximum percentages of indicated positive results within lab varied between 10 and 35% (see Fig. 8).

Reasons for this different interpretation may be: interpretation of colour and/or adjustment of correct reading time. Possibly the 'reproducibility' may increase if other substances besides the most often applied penicillin are used as positive references for fixing the incubation period or if

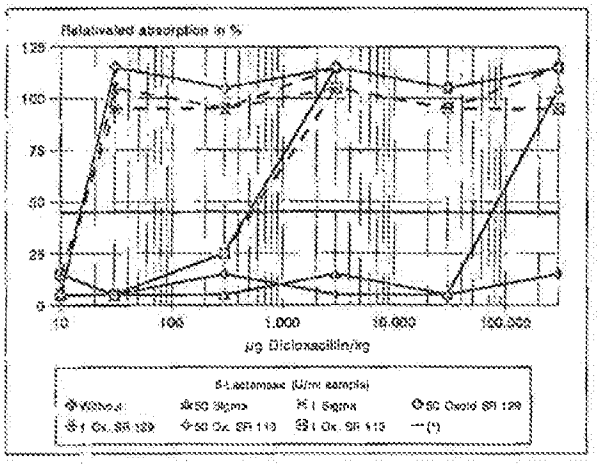

Figure 6. Identification of inhibitors by β-lactamases (Delvotest SP) – Dicloxacillin –
(*) ≥45% relativated absorption positive results

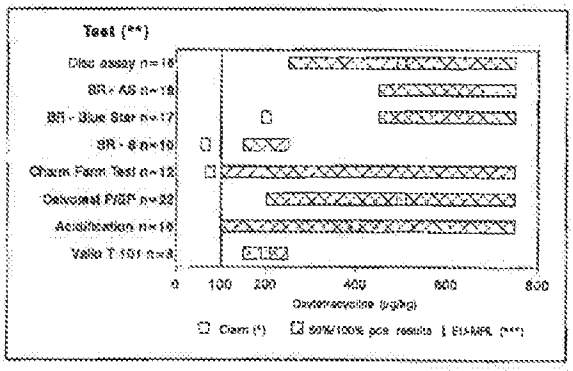

Figure 7. IDF-Intercomparison study 1992 – Oxytetracycline Detection limits [µg/kg] of various microbial inhibitor tests
(*) IDF-Bulletin No. 258 (1991) or claim of the manufacturer;
(**) n = number of subsamples;
(***) all substances belonging to the tetracycline group

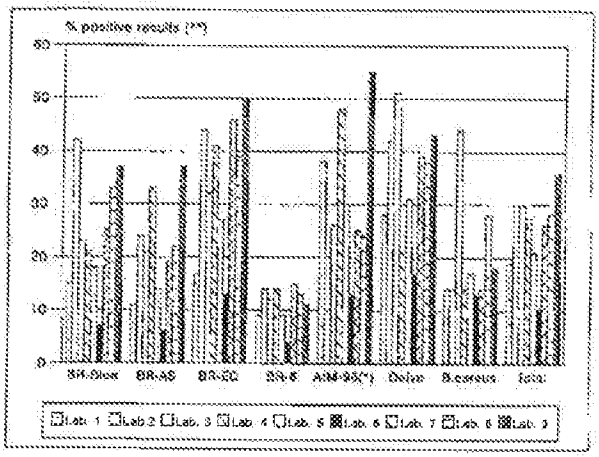

Figure 8. Proficiency study with microbial inhibitor tests. Positive results [%] within test and laboratory
(*) without lab 2; (**) n = 284 within each lab

the subjective visual reading is replaced by objective evaluation by means of an ELISA-reader [37].

Standardized description of microbial inhibitor tests

The microbial inhibitor tests most often applied are proprietary techniques which will not and cannot be described as a standard method — for example IDF-Standard — in such a way that it will be possible for users to prepare the test systems in their own labs with the features described in the standard. Furthermore, due to the situation that the requirements according to changing values and/or substances for which detection limits are fixed a tool is necessary which is more flexible than a standard procedure. It was therefore the decision of IDF-Group E503 not to describe methods like a standard but to develop a test validation concept which will allow the criteria of the method to be described in a standardized form. A first draft of such a checklist for the standardized description of microbial inhibitor tests is in the state of first discussions and includes the items listed in Table 4.

Table 4. Checklist for the standardized description of microbial inhibitor tests IDF-E-503 Rapporteurs: G. Suhren, R. Beukers, J. Reichmuth

1. Summarized information from the manufacturer/inventor
2. Evaluation of the attributes of the test under study
   - Prerequisites for experimental studies
   - Sensitivity — detection limits
   - Variation between batches
   - Variation within batch
   - Shelf life of test kits
   - Sample preservation
   - Identification of inhibitors
   - Susceptibility for interferences
   - Detection of incurred substances
   - Reproducibility between laboratories
3. Standardized description and expert opinion on the applicability of the intended use

Conclusions

As has been demonstrated within this paper, as examples some of the items listed in Table 4, have to be evaluated for each test and antimicrobial in question individually. As microbial inhibitor tests are 'broad spectrum' tests it will not be possible to have a complete examination in every case. A standardized description of the evaluated results should help to make clear what information is available and what is missing in order to facilitate the decision of the user of a test whether and how the microbial inhibitor test fits into his 'demand pattern' with respect to detection limits and field of application.

Acknowledgement

Experimental work presented here was supported in part by a grant of the EU (Contract No. 1116/92-2.1).

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Received 1 November 1995

Charakterisierung pankreatischer Casein-Plasteine

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Zusammenfassung

Im Verlauf der Plasteinreaktion konzentrieren sich hydrophobe Peptide überwiegend in den Aggregaten (Plasteinen), während hydrophile Peptide in Lösung (Überstand) verbleiben. Liquidchromatographische und sequenzanalytische Untersuchungen der pankreatischen Casein-Plasteine haben ergeben, daß die Aggregate überwiegend aus den freien Aminosäuren Tyrosin, Phenylalanin und Tryptophan bestehen. Darüber hinaus enthalten die Plasteine kurzkettige Peptide insbesondere vom C-terminalen Ende des β-Caseins. Die Charakterisierung der funktionellen Eigenschaften der Plasteine hat deutlich gemacht, daß die Aggregation der kurzkettigen Peptide und freien Aminosäuren auf nicht-kovalenten, hydrophoben und ionogenen Wechselwirkungen beruht. In den Überständen aus der Plasteinreaktion konnten Caseinophosphopeptidsequenzen insbesondere aus α_s-Casein bestimmt werden.

Summary

Characterization of pancreatic casein plasteins

In the course of the plastein reaction hydrophobic peptides concentrate mainly in the aggregates (plasteins), whilst hydrophilic peptides remain in solution (supernatant). Liquid chromatographic and sequence analytical studies of pancreatic casein plasteins have shown that the aggregates consist mainly of the free amino acids tyrosine, phenylalanine and tryptophan. Plasteins contain, in addition, short-chain peptides, particularly from the C-terminal of β-casein. Characterization of the functional properties of the plasteins has shown clearly that aggregation of the short-chain peptides and free amino acids is brought by non-covalent, hydrophobic and ionogenic interactions. In the supernatants resulting from the plastein reaction caseinophosphopeptide sequences, in particular from α_s-casein, were determined.

Einleitung

Die Proteolyse umfaßt ein Spektrum von Reaktionen, das über die reine Hydrolyse von Peptidbindungen hinausgeht. So werden während der *in vivo*- und *in vitro*-Verdauung von Lebensmittelproteinen, insbesondere von Milcheiweiß, bioaktive Peptidsequenzen freigesetzt, die weitgehend proteolyseresistent sind und stoffwechselregulierende Eigenschaften aufweisen [1]. Die enzymatische Umsetzung kann durch entstandene Proteolyseprodukte gehemmt [2] und durch Immobilisierung von Proteinasen in ihrer Spezifität verändert werden [3]. Im Rahmen der Proteolyse können geschützte Aminosäuren terminal kovalent an Peptide gebunden werden [4]. Darüber hinaus sind plasteinähnliche Reaktionen, die sich durch chemisch-physikalisch bedingte Aggregation von Proteolyseprodukten und möglicherweise auch durch enzymatisch katalysierte Transpeptisierungs- und Kondensationsreaktionen an Peptiden auszeichnen, auch bei geringen Substratkonzentrationen (5% w/w) meßbar [5]. Es wird vermutet, daß durch Transpeptisierung entstandene Peptide mit einer Sequenz, die in den Substratmolekülen nicht vorliegt, bei höheren Hydrolysegraden zur Hemmung der Enzymaktivität beitragen [6]. Plasteinähnliche Reaktionen während der Proteolyse bedingen eine partielle Fraktionierung der Peptide nach ihrer Hydrophobizität. Dabei konzentrieren sich hydrophobe Peptide in den während der Reaktion entstandenen Aggregaten, wohingegen hydrophile Proteolyseprodukte, wie beispielsweise Caseinophosphopeptide, überwiegend in kolloider Lösung verbleiben [4]. Dieses

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